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I. BACKGROUND AND RATIONALE

I.A. BACKGROUND

I.A.1 HEMOPHILIA B

Hemophilia B is the bleeding diathesis that results from a deficiency of blood coagulation factor IX. The disease is X-linked and affects approximately 1 in 30,000 males. Current treatment of the disease is based on intravenous infusion of clotting factor concentrates, either recombinant or plasma-derived, in response to bleeding episodes. The major morbidity of the disease is arthropathy that results from recurrent spontaneous bleeds into the joints. A major cause of mortality is bleeding into critical closed spaces (e.g., intracranial, retropharyngeal, or retroperitoneal bleeds). The introduction of factor IX concentrates and of home infusion protocols in the 1960s and 1970s represented a major advance in the treatment of hemophilia. However, widespread use of contaminated plasma-derived clotting factor concentrates resulted in a high rate of human immunodeficiency virus (HIV) infection among individuals with severe disease. Seroconversion studies document that most patients with hemophilia were infected between 1978 and 1984 (1). In the US, most patients with severe hemophilia who are over the age of 20 are HIV-positive, and many have already died from complications related to the acquired immune deficiency syndrome (AIDS). The development of recombinant factor IX protein in the 1990s ensured an additional level of safety, but these products have not circumvented all of the problems of protein-based therapies.

Most individuals with hemophilia B have severe disease, with factor IX levels of < 1%. A generation of clinical research in patients treated with clotting factor concentrates has documented that minimal elevations in the levels of circulating clotting factor are sufficient to prevent much of the morbidity of the disease. The most comprehensive data are contained in the Swedish prophylaxis studies summarized in Lofqvist *et al.* (2). Since 1958, most hemophilia patients in Sweden have been maintained on a regimen in which factor is infused on a regular basis rather than in response to bleeds. The doses used have been gradually increased through the years; the current regimen involves infusion of 25–40 U/kg two–three times weekly. The goal of therapy is to maintain trough factor levels >1% of normal. Data from the most recently published summary showed orthopedic and radiographic joint scores of 0 (normal joints) for all patients under the age of 15; radiographic joint scores in the range of 5–20 would be more typical for individuals in this age group treated only in response to

bleeds (3). Thus, the favorable effect on joint disease of keeping levels even marginally higher than baseline is clear.

Before the AIDS era, central nervous system hemorrhage was the most common cause of premature death in patients with hemophilia (4). In several retrospective analyses and in one prospective study, the prevalence of CNS bleeding ranged between 2.6 and 13.8%, with mortality rates between 20 and 50% (5-7) and morbidity rates (including seizure disorder, motor impairment, or mental retardation) of 40–50% in survivors (5). These bleeds can be present with few or subtle premonitory symptoms, and the bleed can impair the patient's ability to treat himself. The literature documents that these bleeds occur predominantly in patients with severe disease (< 1% factor level) (5). Thus, it is reasonable to suppose that raising the levels of factor even slightly (≥ 2% of normal) would improve the chances for avoiding this life-threatening complication of the disease. The incidence of CNS hemorrhage can also be reduced by the use of prophylactic factor infusions (4).

Prophylactic therapy for hemophilia has not achieved widespread acceptance in the US for several reasons. The expense is prohibitive (an average adult patient with severe disease who treats only in response to bleeds spends $50,000–$100,000 on concentrate each year). In addition, in children treated prophylactically who require indwelling intravenous catheters for infusion there is an attendant risk of catheter-related infections (8). In addition to the cost and the inconvenience associated with the need for intravenous infusion, the use of plasma-derived concentrates carries the additional risks of blood-borne disease transmission associated with all blood products. Current viral inactivation procedures have greatly reduced, but not eliminated, this risk (9).

Gene therapy, a recently proposed alternative approach, would potentially allow patients to realize the benefits of prophylaxis, including reduced incidence of CNS bleeding and of hemophilic arthropathy, without the need for indwelling intravenous catheters or frequent factor infusion.

Several features make hemophilia B a good model for gene therapy. The first advantage of hemophilia B as a model for gene therapy is that precise regulation of transgene expression is not required. Therapeutic range is remarkably wide, from ≥ 1% to 150% of normal. It is clear, based on data from infusion of concentrates into patients with hemophilia B, that levels as high as 150% are not associated with ill-effects since the protein circulates as a zymogen (inactive precursor). Second, as stated, even low levels of between 1–5% of normal provide prophylaxis against chronic arthropathy and CNS bleeding. Patients with

levels of 5% have much milder disease and only rarely experience spontaneous bleeding episodes (although they exhibit abnormal bleeding in response to hemostatic challenges such as surgery or trauma). A third advantage of hemophilia B is the availability of large and small animal models for the human disease. Clearly, animal models are a major asset in efforts to establish an experimental basis for gene therapy. In the case of hemophilia, there are well characterized naturally occurring canine models of the disease and genetically engineered hemophilic mice (10-13). Finally, it should be noted that determination of therapeutic efficacy is straightforward and unequivocal in the case of hemophilia B, since plasma levels of factor IX are easy to measure and correlate well with clinical severity of disease.

I.A.2 FACTOR IX GENE AND PROTEIN

The human factor IX gene is located on the X chromosome at Xq26 through Xq27 (14). It spans 35 kb and consists of eight exons separated by seven introns (15). The exons correlate approximately with the functional domains of the protein. Thus, exon 1 encodes the signal sequence, exon 2 the propeptide and γ-carboxyglutamic acid (Gla) domain, exon 3 a short connecting peptide, exons 4 and 5 the first and second epidermal growth factor (EGF)-like domains respectively, exon 6 the activation peptide, and exons 7 and 8 the catalytic domain and the long 3' untranslated sequence. The human cDNA is 3 kb in length, encoding a mature protein of 415 amino acids. The factor IX genes of all species studied to date (including the human gene) contain a long (~ 1.4 kb) 3' untranslated sequence. The precise function of this sequence is unknown, and it has not been included in most recombinant vectors expressing human factor IX.

The biosynthesis of factor IX is complex because of a number of post-translational modifications that are required in order for the protein to have full biological activity (16). These include proteolytic removal of the signal sequence and the propeptide; γ-carboxylation of 12 glutamic acid residues at the N-terminus of the protein in a vitamin K-dependent reaction; and glycosylation at specific residues in the light chain (the N-terminal portion of the molecule) and the activation peptide. The enzyme γ-glutamylcarboxylase, which catalyzes the conversion of specific glutamic acid residues to γ-carboxyglutamic acid, is found in high levels in hepatocytes, but is also present in a variety of other tissues including muscle (17). Biologically active factor IX has been synthesized in a wide range of cells including CHO cells, endothelial cells, myoblasts, and fibroblasts (18-20).

I.A.3 BIOLOGY OF ADENO-ASSOCIATED VIRUS (AAV) VECTORS

Adeno-associated virus is a non-enveloped, replication-defective parvovirus that has not been associated with human disease. AAV vectors are derived from the parent virus by removing all of the viral elements except for the inverted terminal repeats (ITR) and inserting the gene or genes of interest and their associated regulatory elements. The long-term safety of these vectors in humans is unknown; however, initial results of two clinical trials using AAV vectors to deliver the gene for the cystic fibrosis transmembrane regulator (CFTR) directly to the respiratory tract have not resulted in adverse events.

AAV vectors do not require actively dividing target cells to achieve efficient transduction, as demonstrated in post-mitotic cells of brain, liver, and muscle *in vivo* (21-26). Also, because all of the viral genes have been removed, there is no immune response directed against the transduced cell due to viral gene expression. This accounts, at least in part, for prolonged (months to years) transgene expression observed *in vivo* following a single administration of an AAV vector. Several groups have now established that AAV efficiently transduces liver cells following a single administration via the portal vein resulting in long-term, (> 2 years), dose-dependent transgene expression (25-27).

I.A.4 DESCRIPTION OF THE AAV-hFIX VECTOR

The AAV-hFIX vector was derived from the naturally occurring virus adeno-associated virus (AAV) that has not been associated with human disease and is ubiquitous in the environment. The virus consists of a single-stranded DNA genome encapsidated in a protein coat. The AAV genome consists of three elements: the *rep* gene, the *cap* gene and the inverted terminal repeats (ITRs). The *rep* gene directs production of the proteins that enable the virus to replicate its genome. The *cap* gene directs production of the protein coat. These two viral genes are flanked by the ITRs, identical segments of DNA required for replication and encapsidation.

The AAV-hFIX16 vector is derived from wild-type AAV using recombinant DNA techniques and contains a single-stranded DNA genome of 4492 nucleotides. All of the viral genes have been removed and replaced with the following: 1) an expression cassette that contains the human α1-antitrypsin promoter coupled to the human apolipoprotein E enhancer (28) and hepatocyte control region (29) 2) exon 1 from the human factor IX gene; 3) a portion of the human factor IX intron 1; 4) exons 2-8 of the human factor IX gene; and 5) the bovine growth hormone polyadenylation signal sequence. Small intervening non-functional DNA

sequences are derived in the process of assembling the genetic elements through recombinant DNA techniques. The expression cassette is flanked by the 145 nucleotide inverted terminal repeats derived from AAV type 2.

AAV-hFIX16 vector is produced by transfecting Human Embryo Kidney (HEK) 293 cells with plasmid DNA encoding the genes required for vector amplification and packaging. Vector particles are released from harvested cells by freeze/thaw, purified by density gradient centrifugation, and concentrated / diafiltered by tangential flow filtration. Each lot of AAV-hFIX16 is tested for identity, concentration, potency, purity, pH, sterility, and appearance.

I.B. MANUFACTURE

AAV-hFIX16 vector is manufactured in compliance with FDA Current Good Manufacturing Practice (cGMP) Regulations by Avigen, Inc, 1201 Harbor Bay Parkway Suite 1000, Alameda, CA 94502.

II. CLINICAL UPDATE

Based on previously published preclinical efficacy and safety studies in animals, a human clinical trial has been initiated using a rAAV vector to deliver the gene for human factor IX into the liver. The AAV vector containing a gene for human factor IX (AAV-hFIX) is administered into the hepatic artery via arteriography. The study design is open-label, non-randomized, single administration with inter-subject group dose escalations. Up to 10 subjects with severe hemophilia B will be enrolled and assigned to groups of two to four. Subjects within each group will receive the same dose of AAV-hFIX16 vector as indicated in Table 1.

Table 1

Dose Escalation Plan

Group	Subjects	Dose/kg[1] (v.g.)[2]	Total Dose[3] (v.g.)	Observed F.IX levels in mice	Observed F.IX levels in dogs
1	2	2.0×10^{11}	1.4×10^{13}	~1%	-
2	4	1.0×10^{12}	7.0×10^{13}	5%	4-12%
3	4	5.0×10^{12}	3.5×10^{14}	20-30%	-

[1] dosing will be performed according to the patient weight obtained at the time of injection
[2] vector genomes
[3] assuming a 70 kg adult

Table 2

Subject # 1: LFT / PT

	BL	D1	D2	D3	D4	D5	W1	W 2	W 3	W4	W6	W10	W12	W16	W20	W24
ALT (IU/L)	22	9	9	8	21	21	6	9	12	10	9	25	6	12	18	10
AST (IU/L)	22	23	18	22	22	18	20	19	23	18	20	43	19	23	22	23
Alk Phos (IU/L)	86	77	76	80	86	82	77	83	91	83	79	84	77	67	69	67
PT (sec)	12.3	11.2	11.3	11.1	10.6	11.8	11.9	12.3	12.4	12.3	12.1	12.2	11.7	11.5	11.3	11.4

Following vector administration, subjects are evaluated for local or systemic toxicity by clinical and laboratory assessment during the initial hospitalization, the first week following treatment, and then periodically for the first year. Complete blood counts, coagulation studies, and serum chemistries are regularly monitored. In addition, Factor IX inhibitor titers and vector shedding (urine, blood, saliva, and stool) are evaluated. To evaluate the potential for vertical transmission of AAV vector, semen samples are obtained at baseline, during week 1, at months 1–4, and then monthly until 3 serial samples are negative for vector DNA by PCR. The semen samples at two, three and four months (days 60, 90, and 120) are subjected to a fractionation procedure that will allow testing of the motile sperm fraction for vector sequence. Currently, dose escalation cannot take place unless all subjects in the previous cohort have been shown to be free of vector sequence in the motile sperm fraction at day 60. Subjects are counseled to consider banking sperm prior to administration of AAV-hFIX16, and to practice barrier (condom) contraception until three consecutive monthly semen samples are negative.

II.A. SUBJECT 1

To date, two subjects have been enrolled and treated in this study. The first subject had no serious adverse clinical or laboratory events (Tables 2,3) as now observed to Week 24. Vector shedding in various body fluids were determined by a PCR assay with a sensitivity of 10 copies per microgram of total genomic DNA. All semen samples were analyzed in triplicate. Vector sequences were detected in serum out to 14 days, and detected in semen out to 6-10 weeks but have

subsequently been negative (Table 4). Fractionation of the semen sample from week 3 indicated the motile sperm component was found to be negative for rAAV vector sequences while the seminal fluid was positive (Table 5).

Table 3

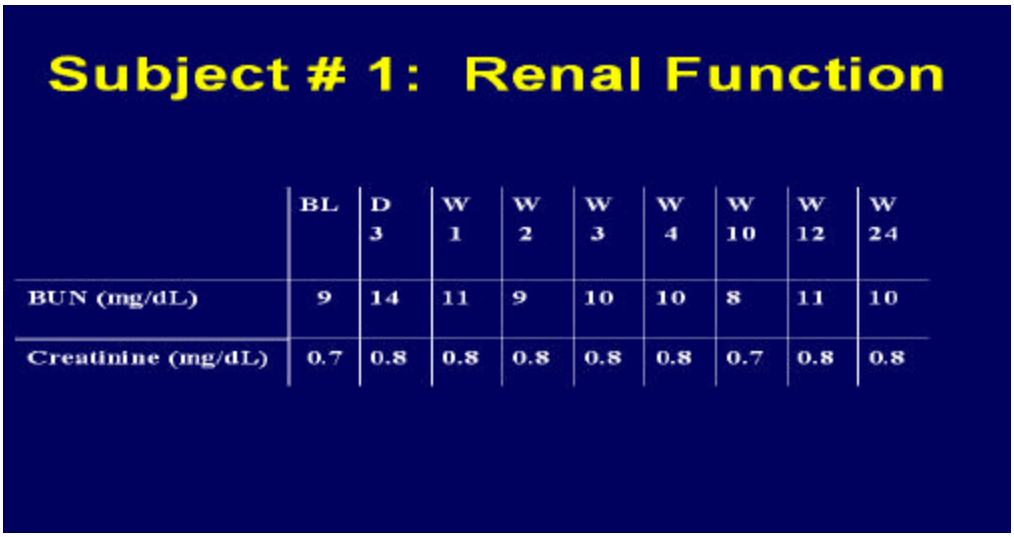


Table 4

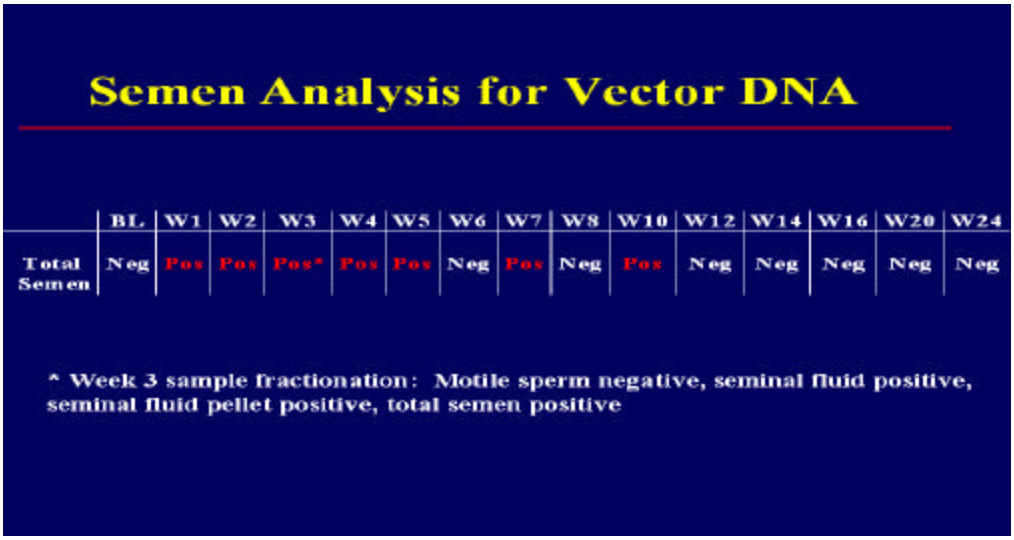
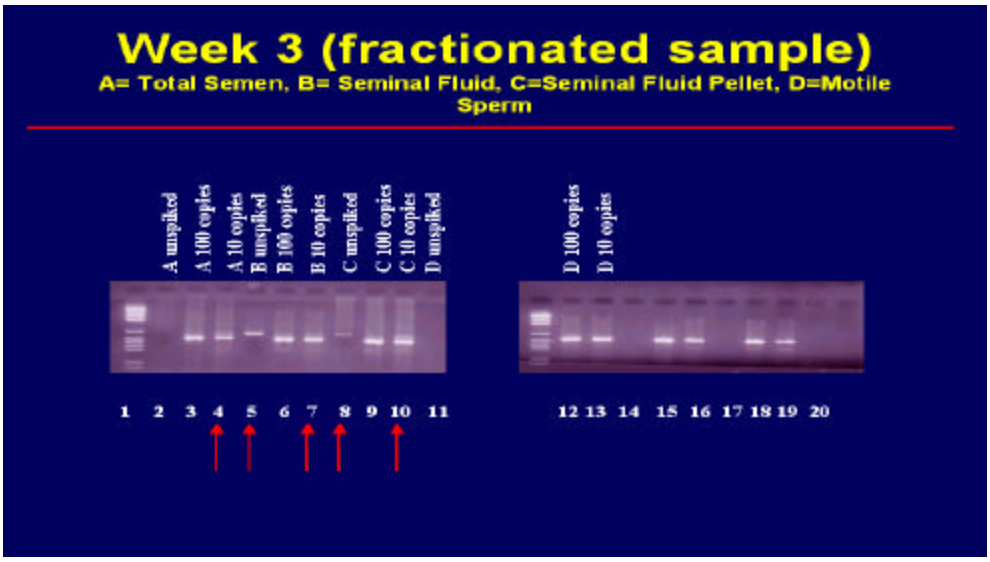


Table 5



II.B. SUBJECT 2

A second subject with severe factor IX deficiency was enrolled into the study approximately 8 weeks ago. The procedure was tolerated without adverse clinical or laboratory events (Tables 6,7). PCR analysis of total semen has determined positive vector sequences in the week 1, 4, and 8 samples. Fractionation of the week 8 specimen could not be performed using the Andrology Laboratory's standard fractionation procedure, due to the low volume of semen obtained (Table 8). PCR analysis of total semen samples to date show a pattern similar to that seen with the first subject, ie. gradually diminishing intensity of positive signals (Table 9).

Table 6

	BL	D 1	D 2	D 3	D 4	D 5	W 1	W 2	W 3	W 4	W 6	W 8
ALT (IU/L)	15	15	19	19	21	18	19	18	19	18	21	17
AST (IU/L)	28	28	44	29	29	31	29	25	27	26	28	28
Alk Phos (IU/L)	65	57	62	70	67	68	69	67	69	71	70	63
Bilirubin	0.6	1.2	1.3	1.5	1.4	1.1	1.1	1.0	1.1	1.1	1.2	0.9
PT (seconds)	11.8	12.6	12.3	12.0	11.7	12.7	11.9	13.1	12.1	12.2	12.1	12.1

Table 7

Subject # 2: Renal Function

	BL	D 3	W 1	W 2	W 3	W 4	W 6	W 8
BUN (mg/dL)	18	16	14	10	13	12	13	13
Creatinine (mg/dL)	0.9	1.0	0.9	0.9	0.8	0.9	1.5	0.9

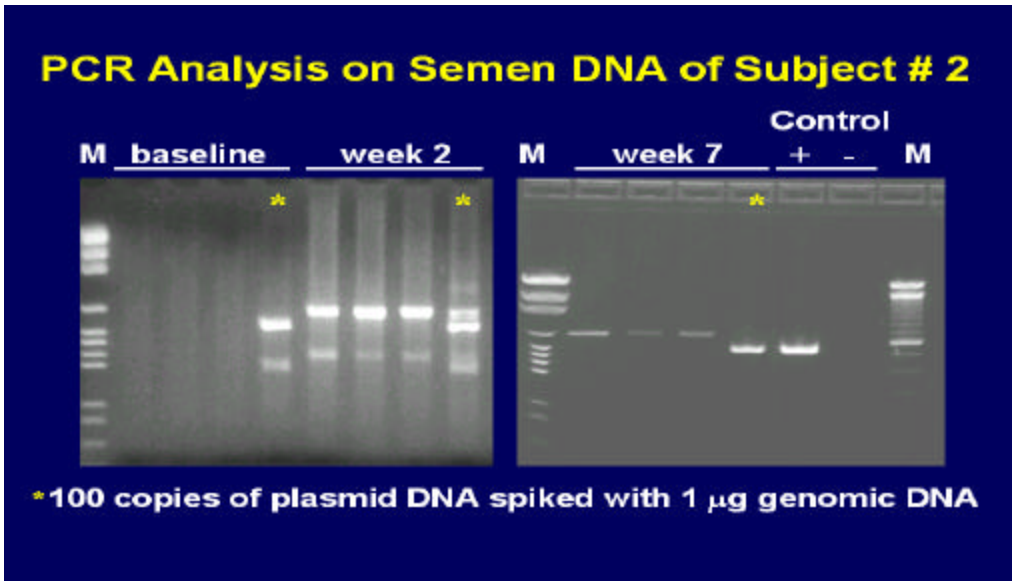
Table 8

Subject # 2: Semen Volume/DNA

	Semen Total Volume (µl)	DNA (µg/mL)	DNA Final Volume (µl)	Total Amount DNA (µg)	Total Amount DNA per PCR (µg)
BL	100	1250	100	125	1
Day 7	100	4869	100	487	1
Week 4	*	8	50	0.4	0.07
Week 7	150	77.4	50	3.85	0.77
Week 8	200	845	200	169	1

*unmeasurable

Table 9



III. PRECLINICAL UPDATE

III.A. PRE-CLINICAL EFFICACY STUDIES

Numerous studies have been performed by a number of investigators (25-27,30-33) to demonstrate the efficacy of delivering an AAV-FIX vector to the livers of normal and hemophilic mice and to hemophilic dogs. Normal mice injected with the AAV-hFIX vector being used in the clinical trial at doses ranging from 1.0 x 10^{11}-7.0 x 10^{13} vg/kg have produced circulating FIX levels of between 50 and 25,000 ng/ml, representing 1-500% of normal. Hemophilic mice injected with a different AAV-hFIX vector at doses of 8x10^{12} - 2.2 x 10^{13} vg/kg resulted in similar plasma FIX levels of 1000-20,000 ng/ml, resulting in the amelioration of the bleeding diathesis in these animal (33). Efficacy has also been demonstrated in hemophilic B dogs following injection of an AAV-cFIX vector. Our data demonstrate that doses of approximately 10^{12} vg/kg resulted in sustained Factor IX levels of 4-12% (30). In addition, Wang et al (32) have seen levels of 218 ng/ml following administration of 4.6x10^{12} vg/kg of an AAV-cFIX vector containing a different expression cassette.

III.B. MOLECULAR CHARACTERIZATION OF VECTOR DNA IN LIVER

AAV gene transfer to the mouse liver has been studied at the molecular level by a number of investigators. Following injection into either the liver or the muscle, expression rises gradually over a period of 2–8 weeks. Using DNA in-situ hybridization it has been demonstrated that most of the hepatic nuclei take up the

vector following portal vein administraton of 3 x 10^{10} to 2 x 10^{11} vg per mouse, but only about 5 percent of the hepatocytes are stably transduced (34). Vector DNA is converted from the single-stranded input form into a double-stranded genome in the stably transduced cells. The molecular structures of the double-stranded genomes represent a complex mixture of circular and linear DNA forms. Some of the double-stranded DNA forms are present as concatemers, with equal numbers of head to head, tail to tail, and head to tail structures (35) An ongoing area of investigation has been the attempt to determine whether these high molecular weight forms are integrated into the host cell genome or persist as episomal forms. Miao et al. (36) have recently provided two types of evidence suggesting that the vector concatemers and/or integrated DNA forms were present following injection into the portal circulation of the mouse. They used fluorescence in-situ hybridization (FISH) analysis performed on metaphase spreads from mouse hepatocytes to demonstrate that a hybridization signal arising from an rAAV probe was found on sister chromatids in metaphase chromosomes. Finally, Nakai et al. (35), isolated rAAV-mouse chromosomal junction fragments from transduced mouse liver. Over 15 of these fragments have been molecularly characterized**.** These early studies using methods that established integration, were not able to quantify the amount of integrated AAV DNA in liver. More recently an in vivo liver regeneration assay was used to demonstrate that in most cases, chromosomal integration of rAAV vector genomes was relatively inefficient (approximately 5%). These studies demonstrated that the majority of gene expression was derived from episomal forms of the vector (37).

III.C. SAFETY AND BIODISTRIBUTION STUDIES

Safety of rAAV delivery to the liver has been evaluated in five animal species: mice, rats, dogs, non-human primates, and most recently rabbits (see Table 1). A GLP toxicology and biodistribution study was performed in mice and rats, a non-GLP study in normal dogs evaluated toxicology and gonadal/semen dissemination of vector, and a GLP toxicology study was performed in non-human primates that also included analysis of dissemination of vector to liver and gonadal tissue (the biodistribution analysis was done in a non-GLP setting). Recently we have demonstrated that rabbits are the most useful species to assess vector distribution to semen and to monitor the kinetics of its clearance.

Table 1 Summary of Safety Studies

Species	Vector	Dose (vg/kg)	Route	Analysis
Mice	AAV-mFIX	$1.7x10^{12}$	IV	Biodistribution (10 tissues)
Rat	AAV-hFIX	$1x10^{11}$-$1x10^{13}$	HA	Toxicology Biodistribution (8 tissues)
Dog	AAV-null	3.7-$7x10^{12}$	HA	Toxicology Biodistribution (liver, spleen, gonads, semen)
Cynomologus Monkey	AAV-hFIX	$7x10^{12}$	HA, PV	Toxicology (IF staining of testes)
Rabbit	AAV-hFIX	$1x10^{11}$-$1x10^{13}$	IV	Biodistribution (gonads, semen, fractionated semen, serum, WBC)

There was no evidence for vector related toxicity in rats, dogs or non-human primates at doses of rAAV up to 10^{13} vg/kg. In this document we will focus only on those aspects of the studies listed which address issues related to potential for inadvertent germ line transmission.

Biodistribution of rAAV vector or in some cases just gonadal dissemination of vector was evaluated in each of the safety studies described above.

A GLP study using mice was performed to evaluate the distribution of vector to tissues, including gonads. Animals were injected intravenously with AAV-murine F.IX at 1.7×10^{12} vg/kg and sacrificed at day 31 or day 91 post-injection. Using genomic DNA obtained from gonadal tissue, a PCR assay revealed vector sequences in all injected animals (n=4) after 31 days. However, at day 91 vector sequences were seen in only 3/4 animals, and the PCR signal was weak in one of these three compared to the intensity in the first cohort of animals (Table 2).

Table 2

Reproductive toxicology results in mice, rats and dogs following delivery of an AAV vector

Animal	# of Animals	Route of Administration	Vector	Vector Genome per kg	Day of Sample Collection*	PCR positive signal # of animals	Vector copy per μg DNA
Mice	10	Intravenous	AAV-CMV-murine F.IX	1.7×10^{12}	31 91	4/4 3/4	100 0-10
Rat	12	Hepatic Artery	AAV-ApoEhAAT human F.IX	1.0×10^{11}	50 92	1/6 0/6	2 Not detected
Rat	11	Hepatic Artery	AAV-ApoEhAAT human F.IX	1.0×10^{12}	50 92	4/6 0/5	1-3 Not detected
Rat	12	Hepatic Artery	AAV-ApoEhAAT human F.IX	1.0×10^{13}	50 92	6/6 4/6	4-16 2-7
Dog	3	Hepatic Artery	AAV-null vector	3.7×10^{12} to 7.0×10^{12}	90**	0/3	Not detected

*Results from gonadal tissue harvested from all animals at the time of sacrifice.

**Semen samples collected for all 3 dogs at 7, 30, 60, and 90 days following injection were persistently negative.

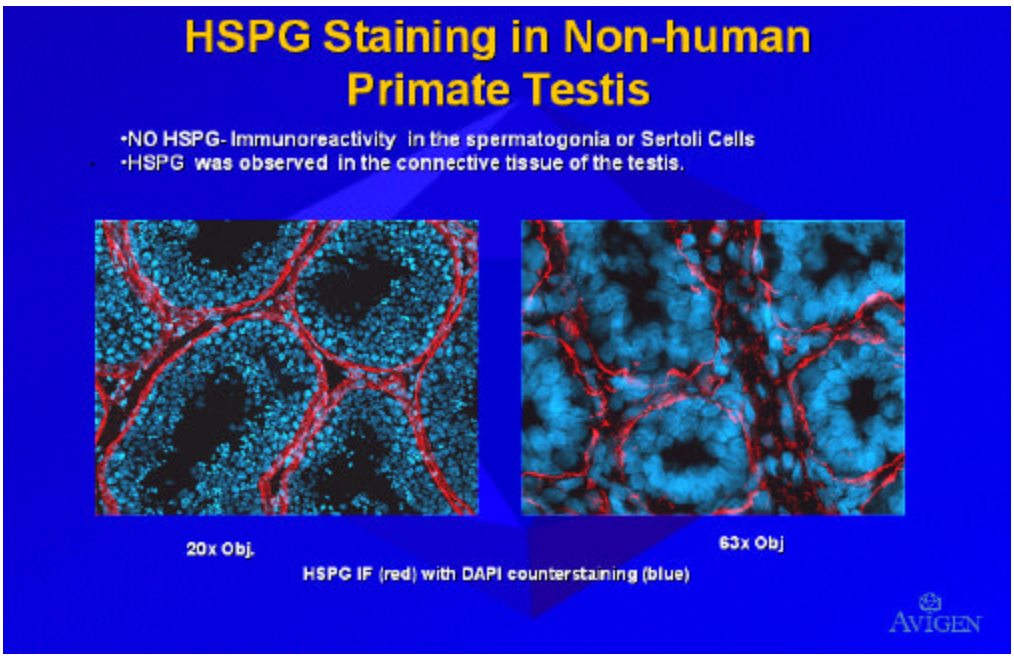
The GLP rat study included quantitative PCR analysis of seven tissues and blood at two time points (days 50 and 92) following vector administration. Three doses of AAV-hFIX ranging from $1x10^{11}$-$1x10^{13}$ vector genomes per kg were used, the highest dose being 50 fold higher than the starting clinical dose. Vector was observed in the testes in animals from all groups, 50 days post injection (Table 2). However, a dose response was observed as more animals in the high dose group were positive. By 92 days post-injection no animals with positive testes were observed in the two lower dose cohorts, indicating clearance of the vector over time. Relative to the target tissue, liver, gonadal tissue had 1000 fold lower levels of AAV vector sequences.

A less comprehensive non-GLP study in dogs evaluated biodistribution of the AAV-Null vector in three tissues (liver, spleen, testes) and semen. In this study, the vector was administered to the hepatic artery using a catheter under fluorographic guidance, similar to the procedure that is being used in subjects. Southern blot analyses detected vector sequences in all four lobes of the liver (0.05-0.27 copies/diploid genome), but not in spleen. Using a PCR assay with a sensitivity of 100 copies per microgram of genomic DNA, vector sequences were not detected in testes 90 days post-injection (Table 2) nor in semen samples collected 0, 7, 30, 60, and 90 days post vector infusion (38). These findings were

used to support delivery of AAV to the hepatic artery using the interventional radiographic procedure, while minimizing the probability of vector dissemination to non-target tissues, such as spleen and gonads.

The GLP non-human primate toxicology study included a non-GLP component to assess vector dissemination to liver and gonadal tissue, however these studies are not yet completed. Following initiation of the study we attempted to collect semen from the animals. After several failed attempts, samples were collected on days 82-89. PCR analysis of DNA extracted from both excipient treated and AAV injected animals was positive. The source of the contamination could not be determined; therefore, we made a decision to sacrifice the animals to collect the histopathology data, which was the primary goal of the study. We subsequently evaluated the testicular tissue using immunofluorescence for the presence of heparin sulphate proteoglycan (HSPG), which has been reported to be the primary receptor for AAV (39). This analysis clearly demonstrates the presence of HSPG in the interstitial space surrounding the seminiferous tubules, but is completely absent from spermatogenic cells within the tubules, suggesting that AAV is unable to infect spermatogenic cells (Figure 1).

Figure 1



Following the discovery of vector sequences in the semen of the initial subjects enrolled in the hepatic artery trial, we evaluated rabbits as a model to study shedding of vector into semen. Rabbits were injected intravenously with AAV-FIX at doses ranging from 1 x 10^{11} to 1 x 10^{13} vg/kg. Semen was collected at time

points ranging from 7 to 87 days following injection. DNA was extracted and analyzed by a sensitive PCR assay that can detect as few as 10 copies of vector DNA in 1 µg DNA. For some experiments, semen was fractionated by density centrifugation and DNA was extracted from the motile sperm fraction and the non-motile sperm fraction. Initial efforts to fractionate rabbit semen using protocols developed for fractionation of human semen showed heavy contamination of the motile sperm fraction with other cells and with non-motile sperm. Revision of the protocol to include centrifugation at higher speeds resulted in a pure motile sperm fraction.

Results show that in rabbits injected at a dose of 1 x 10^{11} vg/kg, DNA extracted from total semen was consistently negative for vector sequences (Table 3). At a 10-fold higher dose, total semen was positive for vector sequences through day 22 and negative thereafter. At 1 x 10^{13} vg/kg, semen DNA was positive for 44 days and thereafter negative. For the analysis of motile sperm, the initial time point in the mid dose group was positive, with all samples negative thereafter. This likely reflects an artifact, since the samples at this time point were fractionated using the protocol developed for human semen.

Table 3

SERIAL TIME POINTS

TOTAL SEMEN ANALYSIS

Dose	7	15	22	38	44	50	87
1 x 11^{11}	0/3	0/3	0/3	0/3	0/3	0/3	0/3
1 x 10^{12}	1/3	2/3	1/3	0/3	0/3	0/3	0/3
1 x 10^{13}	3/3	1/3	2/3	1/3	2/3	0/3	0/3

MOTILE SPERM ANALYSIS

Dose	7	22	50	87
1 x 11^{11}	0/3	0/3	N/S	0/3
1 x 10^{12}	2/3	0/3	Inhibitor	0/3
1 x 10^{13}	N/S	0/3	0/3	0/3

NON-MOTILE SPERM/SEMINAL FLUID

Dose	7	22	50	87
1 x 11^{11}	0/3	0/3	N/S	0/3
1 x 10^{12}	2/3	1/3	0/3	0/3
1 x 10^{13}	N/S	3/3	1/3	0/3

In earlier work, we have shown that AAV vector injected either intramuscularly or into the intravascular space spreads by hematogenous dissemination to the gonads (38, see attached paper). FISH analysis of rabbit testis in these previous studies suggested that vector tends to adhere to the blood vessel walls

and to testicular basement membrane, structures that are rich in heparin sulfate proteoglycan, which binds AAV-2 tightly (39). However, FISH analysis was positive only at 7 days, never at later time points. In addition, attempts to transduce murine spermatogonia directly with AAV-2 in culture were negative (38). The results reported here are consistent with these earlier findings, and suggest that vector that spreads to the gonads by hematogenous dissemination is lost over time, probably by either degradation or by washout.

A major safety issue to be addressed is whether harm may arise from PCR-detectable vector sequences in the semen. The major risks are presumably the risk of horizontal transmission, and the risk of vertical transmission. Studies by others indicate that the risk of horizontal transmission is low. Favre et al. (40) showed using an infectious center assay in non-human primates that had been injected IM with rAAV, that body fluids collected from the animals never contained infectious virions at time points beyond 72 hrs after injection. The risk of vertical transmission will depend on whether vector integrates in motile sperm. From the available data, this would appear to be unlikely since the motile sperm fraction has been negative for vector sequences.

The cumulative biodistribution data indicate that AAV vector disseminates to multiple tissues following hepatic artery administration, but is predominantly found in the target tissue, liver, at a level of 0.6 copies per diploid genome following administration of 1×10^{13} vg/kg. Vector DNA is at least 1000 times less abundant in gonadal tissue. The level of vector sequences in tissue is dose dependent and clears with time. Rabbits have thus far proven the most useful species in studying the significance of vector in semen. Low but detectable levels of vector have been detected in rabbit total semen at early time points following infusion of high doses of vector, but has not been reproducibly observed in motile sperm. Immunofluorescence studies of murine and non-human primate testicular tissue indicates that spermatogenic cells do not express HSPG, the receptor for AAV, and FISH analysis indicates that while AAV sequences are associated with gonadal tissue, they remain bound to the testicular basement membrane. Furthermore, direct exposure of mouse sperm cells to high concentrations of AAV failed to result in transduction. These data suggest that germline transmission of AAV is unlikely, but additional experiments are underway to assess the kinetics of vector shedding in semen and to determine the cellular distribution of AAV in semen.

III.D. IMMUNE RESPONSES AGAINST THE EXPRESSED COAGULATION FACTOR

The risk of inhibitor formation from protein- based therapies of the hemophilias is lower with hemophilia B than with hemophilia A. High and colleagues (The Children's Hospital of Philadelphia) have carried out detailed studies of the immune response against Factor IX following gene and protein-based treatment in both hemophilic and normal mice (41,42). Intramuscular administration of AAV-hFIX vector in hemophilia B mice on a C57Bl/6 background shows that human FIX elicits a T cell response in these mice, with neutralizing antibody formation by day 14, while murine FIX results in a less marked T cell response with reduced titers of neutralizing antibody that are not detectable until 4 weeks after injection. Anti-murine FIX titers are much higher in hemophilia B mice on a CD-1 background, indicating that strain variations influence immune response. Since all these animals have a complete null mutation, data obtained with a human FIX transgene are likely just as valid as data obtained with murine FIX.

Intramuscular administration of AAV-human FIX into hemophilia C57Bl/6 mice results in a neutralizing inhibitory antibody response, primarily of IgG_1 subclass, as expected for a secreted protein. Intravenous infusion of recombinant human FIX (two infusions, two weeks apart) causes a similar IgG_1 response. Therefore, IM injection of vector and IV infusion of protein give similar results in these animals.

Portal vein infusion of AAV-human FIX vector in these mice gives a weak, non-neutralizing antibody response (IgG_{2b}) that can be detected by Western blot or ELISA but not by Bethesda assay. Thus, liver-directed gene therapy may be less immunogenic in C57Bl/6 mice than is protein infusion. While these results are certainly influenced by choice of mouse strain, and cannot be extrapolated to humans directly, they nonetheless suggest that liver-directed gene therapy is not more immunogenic than protein infusion. It is worthwhile to note that hemophilia B dogs with a CRM-negative missense mutation injected with up to 4.6×10^{12} AAV particles did not develop inhibitors against the canine transgene product (31,32).

Additional studies were done in a colony of hemophilia B dogs maintained at Auburn University. These animals have a 5bp deletion followed by a single base change that results in a frame shift and early stop codon leading to an unstable transcript (43). These animals have been found to be more prone to inhibitor formation. Some of these animals have an additional genetic defect related to their gene for red cell pyruvate kinase (PK) leading to a hemolytic anemia, and

extensive iron deposition in the liver. Dogs were infused with vector via the mesenteric vein with doses of AAV-canine F.IX ranging from 1.2-3.4 x 10^{12} vg/kg. A single dog with a combined PK/Hemophilia B lesion developed an inhibitor following an infusion of 3.4 x 10^{12} vg/kg into the portal vein. Two additional dogs without the PK mutation, and one Hemophilia dog with a missense mutation (Chapel Hill colony) did not develop inhibitors following vector delivery at doses up to 1.2 x 10^{12} vg/kg via the portal vein, although high levels of factor IX expression (up to 700 ng/ml) were observed (30). Whether the sole case of inhibitor development following portal vein infusion was a function of dose of vector or underlying PK deficiency (and resulting hepatic iron overload) is not clear at this time.

In the hemophilia B mice, neither portal vein nor IM administration of the AAV vector causes inflammation or activation of cytotoxic T lymphocytes.

III.E. SUMMARY OF PLANNED STUDIES

The studies described below have been initiated to evaluate the potential for both horizontal and vertical transmission of AAV vectors.

Development of an Infectious AAV Assay

Since detecting AAV sequences in the semen of the first two subjects enrolled in the hepatic artery trial, an unresolved question that remains is whether these sequences represent infectious AAV. To address this we have developed a cell based assay to detect infectious AAV in semen samples. The assay requires 200 µl of semen and has a sensitivity of approximately 100-1000 units. Once optimized, the assay will be used in the experiment using rabbits (described below) and in human semen samples.

Rabbit Study

Studies described above have demonstrated that rabbits are a good model to study gonadal distribution of AAV following intravenous delivery. Because the initial rabbit studies did not answer all the relevant questions regarding gonadal distribution, another study has been initiated. This study is designed to determine a) the kinetics of vector shedding in semen, and the persistence of vector DNA in serum, and white blood cells, b) which fraction of the semen contains vector sequences, and c) whether the vector found in the semen is infectious. Twelve rabbits have been injected with 1x10^{11}, 1x10^{12}, or 1x10^{13} vg/kg (4 animals per group). Semen will be collected at weekly time points, and DNA will be extracted

from a portion of the total semen for PCR analysis. A portion will be fractionated into motile sperm, seminal fluid and other cellular components, and fractions will be analyzed by PCR. A third portion will be analyzed for infectious vector using the assay described above. In addition, both serum and WBC will be analyzed for vector sequences.

Direct exposure of mature sperm to AAV

In collaboration with Dr. Jon Gordon, Mt Sinai School of Medicine, we have initiated a study designed to evaluate the potential for vertical transmission of AAV. In this experiment, mouse sperm will be exposed to high doses of AAV-hFIX and then used for in vitro fertilization. The two stage embryos will be implanted into pseudopregnant females and fetuses will be harvested shortly before birth. DNA will be extracted from the fetuses and analyzed for vector DNA by Southern blot analysis. Detection of single copy AAV in the mouse genomic DNA will be used as evidence of germ line transmission.

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